PATENT NO EP (UK) 0529300

TRANSLATION OF EUROPEAN PATENT (UK) UNDER SECTION 77 (6) (a)

Patents Form 54/77

Patents Act 1977
(Rule 80 and Schedule 4)

The Patent Office

Filing a translation in connection with a European patent or a European patent application

(See the notes on the back of this form)

The Patent Office
Cardiff Road
Newport
Gwent NP9 1RH

1. Your reference — GWS/20627

2. European patent number or publication number of application *(or International publication number (see note (e))* — European Patent (UK) 0 529 300

3. Full name and address of the or of each applicant for or proprietor of the European patent (UK) — Dr Rentschler Biotechnologie GmbH
Erwin-Rentschler-Strasse 21
D-88471 Laupheim
Germany

   Patents ADP number *(if you know it)*

4. What kind of translated document listed at note (c) are you sending with this form? — 1(i)

   (Answer by writing 1(i), 1(ii), 1(iii) or 2)

5. Date when the European patent (UK) was granted or amended
   (See note (f))

6. Full name, address and postcode in the United Kingdom to which all correspondence relating to this form and translation should be sent — Mathys & Squire
100 Grays Inn Road
London
WC1X 8AL

   Patents ADP number *(if you know it)* — 1081001

7. Do you want the address in part 6 above to be the address for service recorded on the Register or to replace the address for service currently on the Register?
   (If so then write 'YES') — YES

8. Signature — Date

   Mathys & Squire
   MATHYS & SQUIRE 18 August 1998

9. Name and daytime telephone number of person to contact in the United Kingdom — George W Schlich (0171 830 0000)

0470 SB2

ast Language Services
UNIT 6 · AYR STREET · NOTTINGHAM · NG7 4FX · UK
TEL: +44 (0)115 970 5633 · FAX: +44 (0)115 978 0130

PATENTS ACT 1977

and

PATENTS RULES 1990

I, Andrew William John SCHLICH MA (Oxon),
principal translator and proprietor of AST Language Services, of Unit 6, Ayr Street, Nottingham, England, hereby declare that I am conversant with the German and English languages and that to the best of my knowledge and belief the accompanying document is a true translation of the text on which the European Patent Office intends to grant or has granted European Patent No. 0 529 300
in the name of Dr Rentschler Biotechnologie GmbH.

Signed this 14th day of August 1998

A.W.J. Schlich.

The invention concerns a new recombinant fibroblast interferon, methods for its production and pharmaceutical preparations containing this recombinant human-interferon-beta (IFN-beta).

Naturally occurring interferons are species-specific proteins, mostly glycoproteins, produced by various cells after induction by viruses, double-stranded RNA, other polynucleotides, antigens or mitogens. The interferons have numerous biological activities. The most important of these are antiviral, antiproliferative, immuno-modulating and anticellular effects. Three types of human interferon are recognised which are produced in leukocytes, lymphocytes, fibroblasts and within the immune system. They have been named as alpha-, beta- and gamma-interferons.

Natural human-IFN-beta is produced by induced human fibroblast cell cultures and isolated from the cell culture supernatant and purified. Proteins or polypeptides with human IFN-beta activity can also be produced by the use of the recombinant DNA technique in micro-organisms or eukaryotic cell systems, such as mammalian cell cultures, cf. William E. Stewart II, The Interferon System, 2nd edition, Springer Verlag, Vienna, New York (1981); EP-A 41 313; W. Reiser and H. Hauser, Arzneim.-Forsch./Drug Res. 37 (1) No.4 (1987), page 482; McCormic et al., Molecular and Cellular Biology, Vol. 4, No. 1, page 166 (1984); EP-A-O 388 799; Chernajovsky et al., DNA, vol.3, No. 4, page 297 (1984).

Natural and recombinant interferons have to date been used with varying success in the treatment of patients with oncological, viral, and auto-immune diseases. An anti-neoplastic response in between 20 and 40% of patients with Kaposi's sarcoma related to AIDS in its early stages has been achieved with a variety of alpha-interferons. Disadvantages of alpha-interferon, however, are haematological intolerance and hepatic complications, which place a limit on the size of dose which may be administered. Another disadvantage of IFN-alpha treatment and concomitant administration of zidovudine is that both substances exhibit a high level of haematological toxicity so that concomitant administration results in intolerable side effects and is therefore ruled out (cf. Annals of Internal Medicine, Vol. 112, No.3, 582 (1990).

Fibroblast interferon (IFN-beta) has many immunological properties which are comparable to those of IFN-alpha. However, IFN-beta is markedly better tolerated and presents demonstrably lower haematological toxicity than IFN-alpha in the cases studied thus far of kidney cell carcinoma and lymphoid tumours. In studies in vitro, IFN-beta inhibits the HIV virus and, with azidothymidine, it displays a synergistic antiretroviral activity.

As resistance to azidothymidine alone develops in long-term administration, it would be highly advantageous to have available substances like IFN-beta, which differ in the ideal case from azidothymidine in terms of antiretroviral activity, mechanism of action and toxicity profile.

A further disadvantage of the interferons, in particular the recombinant preparations, is their instability. In particular the highly purified interferon preparations for clinical use show rapid loss of activity in both liquid and lyophilised forms. For interferons, which have been produced by recombinant DNA technology in bacteria, a further disadvantage is that interferons produced in this way are virtually insoluble in water.

In the current state of the art, numerous proposals of ways to surmount these disadvantages are known, in which most of these procedures propose the addition of various chemical compounds to the interferon preparation, to overcome solubility and/or stability problems.

All such procedures still have the disadvantages, however, in that an additional stage is imposed on the production of interferon preparations, that stability remains unsatisfactory and that many of the additives are not authorised in medicines, either because they possess known toxic effects or because their toxicology has not yet been elucidated, precluding their clinical administration; cf. EP-A 270 799, EP-A 89 245, EP-A 217 645, EP-A 215 658, WO 89/05158, WO 89/02750, EP-A 82 481, EP-A 80 879, US-PS 44 83 849 and WO 90/03784.

The inventor has now surprisingly found that, with an IFN-beta whose production is described hereinafter, not only is an unexpectedly improved anti-tumoral effect achievable, but also more stable liquid or solid (lyophilised) preparations can be produced than is possible with known state-of-the-art preparations.

According to the invention, a new recombinant IFN-beta with the characteristics of Claim 1, methods for its production and stable pharmaceutical preparations of IFN-beta in liquid or lyophilised/solid form, are made available, containing high levels of highly purified recombinant IFN-beta without any pharmacologically unfavourable additives. The IFN-beta according to the invention exhibits a greater degree of anti-tumoral activity, in particular in Kaposi's sarcoma, than that of IFN-beta preparations of natural or recombinant IFN-beta tested to date.

According to the invention, the new IFN-beta is produced using the recombinant DNA-technique in CHO-cell cultures and by a method consisting of a combination of phase distribution-, affinity-, metal chelation- and size exclusion chromatography.

The recombinant human IFN-beta according to the invention is characterised by:

(a) a specific glycosylation pattern;

(b) high volume activity of $> 40 \times 10^6$ IU/ml;

(c) high degree of intrinsic stability;

(d) a higher degree of clinical activity, in particular greater anti-tumoral activity

For the production of IFN-beta according to the invention, transduced cells of the CHO (Chinese hamster ovary) line with the laboratory number BIC 8622 (construction of the animal cell line, cf. European Patent Application no. 0 287 075), in the usual cell culture medium (Modified Eagles Medium [MEM]) with Earles salts), supplemented with 0 to 5% foetal calf serum (FCS), are brought to confluence in stationary culture. The cell multiplication constitutes between 1×10^8 and 2×10^9 international units (IU) per day and per litre culture supernatant.

According to the invention the IFN-beta is further enriched by liquid/liquid phase extraction, using an aqueous 2-phase system based on polyalkylene glycol/dextran or polyalkylene glycol/salt.

Of the polyalkylene glycols, polyethylene glycol and/or polypropylene glycol can be used. Of the polyethylene glycols, polyethylene glycols with a MW of 1000 to 6000, preferably 1200 to 3000, most preferably 1500 to 2000 can be used. The concentration lies in the range of 1 to 30% by weight, preferably 2 to 15% by weight, most preferably 3 to 9% by weight. Of the polypropylene glycols, those with an MW of 1500 to 4000 can be used. The concentrations correspond to those for the polyethylene glycols. As dextran, dextrans with a molecular weight of 15,000 to 5,000,000, preferably 100,000 to 1,000,000 most preferably between 350,000 and 550,000 may be used. The concentration lies in the range of 1 to 10 % by weight, preferably 2 to 6% by weight and most preferably in the range of 4 to 6% by weight.

As salts, NaCl, LiCl, NaJ, KJ, NA_2SO_4, NA_2HPO_4, K_2HPO_4, K_2SO_4, NaH_2PO_4, KCl, NH_4Cl, $(NH_4)_2SO_4$, sodium citrate and sodium oxalate alone or in mixtures may be used. The concentration of the salt lies in the range of 2 to 30% by weight, preferably 3 to 20% by weight and most preferably in the range of 4 to 16% by weight.

The cell culture medium containing the IFN-beta is, together with the ingredients of the described aqueous phase system, intensively stirred for 5 to 24h, preferably for 8 to 16h, most preferably for 10 to 12h at between 4 and 25°C, preferably between 10 and 20°C and most preferably between 13 and 18°C and, after demixing and separation, accumulated in the upper phase containing the polyalkylene glycol.

Separation of the aqueous phases occurs by gravitation or other separation technique.

The upper phase containing the polyalkylene glycol contains recombinant IFN-beta at a concentration of between 1×10^8 and 10^{10}/litre. The specific activity is between 5×10^5 and 3×10^7 IU/mg protein. The yields reach almost 100%.

For the final affinity chromatography, blue-dextran-Sepharose®, or other suitable matrices immobilised with Cibacron® blue such as Matrex Gel Blue A ex Amicon or Fraktogel TSK AF-blue ex Merck or Blue-Sepharose® 6FF ex Pharmacia are used.

The upper phase from the phase system is brought to a concentration of between 0.5 and 1.0 mol/litre with NaCl and added to the affinity column at a flow rate of between 1 and 5 cm/min. After washing with PBS, pH 7, or PBS containing 1 to 30% by weight ethylene glycol and/or 1 to 15% propylene glycol, it is finally eluted with PBS containing 10 to 70% by weight ethylene glycol and/or 20 to 50% by weight propylene glycol.

After this step, the recombinant IFN-beta has a concentration of 6×10^6 to 9×10^7 IU/ml eluate and a specific activity of 50×10^4 to 140×10^6 IU/mg protein. The yields amount to between 70 and 90%.

For the metal chelation chromatography, various matrices with chemically identical or differing ligands are suitable. The metal ions suitable for coordinative binding of recombinant IFN-beta can be Cu^{2+}, Zn^{2+}, Co^{2+} or Ni^{2+} ions. Desorption can be effected using competitive substances such as imidazole, histidine, glycine or NH_4Cl, chelating agents such as EDTA, IDA (iminodiacetic acid), TED (tris-carboxymethylethylene diamine) or by lowering the pH value to between pH 2 and pH 4.

Suitable separating media are immobilised iminodiacetic acid coupled to agarose or to Fraktogel TSK HW-65F ex Pierce or Chelating Sepharose® FF ex Pharmacia or Cellufine ex Amicon.

The chromatography medium is added to a suitable chromatography column and loaded with the corresponding metal cation by pumping a solution of this metal ion through the column. After equilibration with a suitable buffer, e.g. PBS, containing 0.1 to 1.0 mol/litre NaCl, the eluate is added to the column at a flow rate of between 0.75 to 1.5 cm/min. The column is finally eluted with a gradient of between 0 and 100 mmol/l imidazole in PBS. Alternatively, a pH gradient of pH 7 to pH2 produced by

mixture of buffer A (PBS; pH7) and buffer B (0.2 mol/l glycine/HCl; pH2) can be used for elution. The isocratic elution with PBS/glycine/HCl buffers with falling pH values is also possible.

After this purification step the recombinant IFN-beta is present at a concentration of between 50 x 10^6 and 300 x 10^6 IU/ml eluate and the specific activity is 150 x 10^6 IU/mg to 220 x 10^6 protein. The yields are between 60 and 90%.

For the size exclusion chromatography various separating media are suitable, which have a fractionation range between about 1000 and about 600 000 Daltons. For example Sephadex® G150, Superfine G150, Sephacryl® S-200 High Resolution, Superose® 12 prep grade ex Pharmacia or TSK-SW 3000 ex Toyo Soda may be used.

The chromatography medium is added to a suitable chromatography column and after settling it is equilibrated with a suitable buffer. As buffer, solutions of PBS with 0 to 0.5 mol/l NaCl are used. The eluate from the metal chromatography is added at a flow rate of 0.5 to 0.8 cm/min. The chromatography ensues with PBS as the mobile buffer containing 0 to 0.5 mol/l NaCl.

After this purification step the recombinant IFN-beta is present at a concentration of between 15 x 10^6 and 50 x 10^6 IU/ml eluate and has a specific activity of between 200 x 10^6 and 300 x 10^6 IU protein. The yields obtained are between 60 and 80%.

The material obtained can finally be further processed or stored at -20°C.

All purification steps are carried out at a temperature of 4 to 15°C.

The expert opinion at the time of publication has been that glycosylated and non glycosylated interferon-beta were equivalent (cf. M. Hawkins et al., Cancer Research 45 (1985) p.5914-5920).

The inventor has found, however, that IFN-beta produced as described above must have a high level of branched carbohydrate chains, biantennal, triantennal, triantennal with at least one repeat and tetraantennal, as well as high levels of sialic acid and fucose if the problem presented is to be solved.

It was found, that the recombinant IFN-beta according to the invention presents a proportion of biantennal oligosaccharide structures of at least 70% and preferably 75%. A further essential ingredient of the recombinant IFN-beta is a proportion of triantennal structures, possibly with at least one repeat linking 1->4 or 1->6, and in which the proportion of triantennal structures is at least 20% and preferably 25%. The recombinant IFN-beta according to the invention also contains a proportion of 0 to 5%, preferably 0.5 to 3%, tetraantennal oligosaccharide structures.

The recombinant IFN-beta according to the invention presents, in its total oligosaccharide moiety, a sialic acid level of at least 90%. The sialic acid moiety is composed of between 90 and 100% N-acetylneuraminic acid and 0 to 10% N-glycolylneuraminic acid.

The IFN-beta according to the invention also contains fucose. Although the fucose content is obviously not essential to product stability, it is present at a rate of 85%, preferably 90% and most preferably >95%.

The specific sugar radicals correspond to the structural formulae presented in the claims. In comparison to IFN-beta from fibroblasts, for example FS4 cells or other CHO cells or other production methods (Chernjovsky et al., DNA 3 (1984), 297-308; Conradt et al., J. Biol. Chem., 262 (1987), 14600-14605; Kagawa et al., J. Biol. Chem. 263 (1988), 17508-17515; Utsumi et al., Eur. J. Biochem. 181 (1989), 545-553; EP-A-0 388 799), the IFN-beta according to the invention possesses a high degree of antennality together with a high degree of sialisation.

The invention also concerns pharmaceutical preparations which contain recombinant IFN-beta with the characteristics described above in combination with the known and usual vehicles and excipients.

High dosage preparations also form part of the preparations according to the invention, i.e. preparations containing between about 18 x 10^6 and about 50 x 10^6 IU, preferably about 20 x 10^6 to about 40 x 10^6 and especially preferably about 25 x 10^6 to 30 x 10^6 IU IFN-beta.

Vehicles which may be used include, for example, an aqueous buffer solution in which the pH value of the solvent is of a physiologically acceptable, preferably neutral, range, i.e. in a range from about 4 to about 8.

It is not necessary to use pH values < 4 or > 8 to dissolve the active substance. Likewise there is no need for the addition of solubilising substances such as SDS or other detergents.

Fillers are merely required, preferably a serum protein such as HSA (human serum albumin) or other known fillers such as PVP.

The pharmaceutical preparations according to the invention can be used for the treatment of tumours, viral diseases, immunopathies or inflammations including rheumatic disorders, allergies, psoriasis, Crohn's disease and degenerative disease of the nervous system, e.g. multiple sclerosis.

The required amount of the recombinant IFN-beta (referred to below as the active substance) to be used in pharmaceutical products for the desired therapeutic effect depends upon the mode of administration and upon the patient as well as upon the disease involved. A suitable dose of the active substance for administration to the human is between about 0.1 x 10^6 and 100 x 10^6 IU. The most advantageous dose for local application is about 0.1 $\leq$ 10^6 to about 6 x 10^6 IU, for systemic treatment about 1 x 10^6 to 30 x 10^6 IU per day, possibly several times a day.

Although it is basically possible to administer the active substance alone, it is recommended that the active substance should be administered in the form of a pharmaceutical formulation, containing the active substance in a pharmaceutically

acceptable vehicle. Usually the active substance in such a form is present at a rate of 1 x 10^6 to 50 x 10^6 IU/ml of solution. For local administration the active substance is present at a rate of about 0.1 x 10^6 to 10 x 10^6 IU/g of preparation.

The pharmaceutical preparations according to the invention contain the active substance in combination with a pharmaceutically acceptable vehicle and possibly further therapeutic substances. The vehicle must be acceptable in the sense that it is compatible with the other ingredients in the formulation and gives rise to no side effects in the patient receiving the formulation.

Formulations are available as appropriate in the form of an ophthalmological, subcutaneous, intracutaneous, intramuscular, intravenous, intrathecal, intra-articular, intratumoral/peritumoral, intralesional/ perilesional or topical preparation.

The formulations according to the invention can be produced by all of the known methods in the field of pharmaceutical technology. Essentially all methods contain the step of combining the active substance with the vehicle and, if required, with one or more additional ingredients. In general the formulations are produced by the even and thorough mixing of the active substance with a liquid vehicle or a fine-particle solid vehicle or both and, finally, if required, forms of the product in the desired mode of preparation.

The formulations according to the invention can be prepared in the form of discrete units, whereby every form contains a specific amount of the active substance. They can also be prepared in the form of a powder or in the form of a granulate or in the form of a solution or in the form of a suspension in an aqueous or nonaqueous liquid or in the form of an oil-in-water emulsion or water-in-oil emulsion. The active substance can also be prepared in the form of a bolus, a linctus or a paste.

If the formulations according to the invention are designed for parenteral administration, they usually contain a sterile aqueous preparation of the active substance, preferably isotonic with the patient's blood. The formulations according to the invention for parenteral administration can also be prepared in the form of a

powder in a sterile vial, e.g. as a lyophilisate, whereby the active substance is prepared by solution in water for injections immediately prior to use.

Preparations according to the invention which are appropriate for intra-articular administration may be in the form of a sterile aqueous preparation of the active substance, whereby the active subjects may be present, if required, in microcrystalline form, e.g. in the form of an aqueous microcrystalline suspension.

The formulations according to the invention may also be prepared in the form of a liposomal preparation or in the form of a biodegradable polymer system for administration of the active substance.

Formulations according to the invention which are suitable for local administration include liquid or semiliquid preparations such as, for instance, embrocations, lotions, dressings, oil-in-water or water-in-oil emulsions such as, for instance, creams, ointments or pastes, or solutions or suspensions such as, for instance, drops. The active ingredient can for instance for ophthalmological administration be prepared in the form of aqueous eyedrops, e.g. in the form of a 0.1 to 1.0% solution.

In addition to the ingredients already described, the formulations according to the invention may contain one or more further usual and known components such as, for example, diluents, buffers, binders, surface active agents, thickeners, lubricants, preservatives, antioxidants, emulsifiers and other such substances.

The IFN-beta used here was produced by the method described in EP-A 287 075, which is expressly referred to here.

The method according to the invention for the production of pure recombinant IFN-beta yields very reproducible IFN-beta with a carbohydrate composition which conforms to the above-mentioned characteristics. The variability is very slight. The method also leads to a highly pure IFN-beta which, because of its high level of activity per volume of 12 to 50 x 10^6 IU/ml in the end product, meets the requirements for a high-dose formulation.

This highly concentrated recombinant IFN-beta presents, unlike less pure IFN-beta with a lower level of activity per volume, a significantly heightened intrinsic stability. This good stability is achieved in buffered saline at neutral pH without the addition of stabilisers of the kind required in state-of the art technique.

The preparations according to the invention exhibit a specific activity of at least 2×10^8 IU/mg. The purity of the IFN-beta used corresponds to at least > 98%, preferably > 99%.

The specific activity is determined by comparison of its antiviral activity with that of an NIH-reference standard. The protein concentration is determined by the standard method according to Lowry.

The following examples serve to illustrate the invention, without however limiting its scope.

Example 1

The production of recombinant IFN-beta

For the construction of a cell culture pyramid with CHO BIC 8622 cells, starting with an ampoule, the cells are transferred stepwise into larger production vessels and multiplied up to an appropriate scale for recombinant IFN-beta production. For the construction of the production pyramid, Roux bottles, double reservoirs, reservoir units and battery reservoir units are used. The harvesting phase takes place in the battery reservoir unit. The medium consists of MEM alpha minus, L-glutamine (2mM) and gentamycin (50 µg/l). To achieve higher yields, 1% to 5% foetal calf serum may be added. Harvesting of 80 l conditioned medium with 1 x 10^5 to 1 x 10^6 IU/l recombinant IFN-beta takes place very 24 hours.

The recombinant IFN-beta presents at this stage a specific activity of 1.0 x 10^6 IU/mg protein.

For phase distribution 80 l conditioned medium are mixed with 80 l of a mixture containing 60 l double-distilled water, 8000 g NaH_2PO_4 x $2H_2O$, 30 000 g K_2HPO_4, 13000 g NaCl, 9000 g polyethylene glycol PEG 2000, in a stainless steel kettle, whereby the mixing temperature is 15°C. After a separation time of 12 hours, the lower phase is disposed of and the supernatant, ca 20. l, retained for further purification. The yield of recombinant IFN-beta amounts almost to 100%.

At this stage, the recombinant IFN-beta displays a specific activity of 3 x 10^6 IU/mg protein.

The ensuing chromatographic purification stages can be carried out at 15°C on the basis of the high intrinsic stability of the recombinant IFN-beta.

The chromatographic purification method begins with affinity chromatography using Blue Sepharose FF (Pharmacia). For this, 0.5 l Blue Sepharose FF is packed into a 10-

cm diameter chromatography column and equilibrated with 1.5 l PBS. The gel bed height is ca. 6 cm. The supernatant, 20 l, is diluted 1:1 with PBS containing 1 mol/l NaCl and is pumped through the column at a flow rate of 3 cm/min. Under these conditions, IFN-beta is 95% bound.

There follows a wash with 2.0 l PBS containing 1 mol/l NaCl. Finally elution with a gradient of 1.0 l PBS and 1.0 l PBS containing 30% w/w propylene glycol and 10% w/e ethylene glycol is performed. The flow rate of the wash and elution is 4 cm/min. In the wash ca. 10% to 15% of interferon activity, in the elution ca. 80% of interferon activity are obtained. The recombinant IFN-beta at this stage has a specific activity of 130 x 10^6 IU/mg protein. The next step is metal chelation chromatography.

For this, a chromatography column of 8 cm. diameter is packed with 0.25 l chelating Sepharose (Pharmacia). The gel bed height is ca. 4 cm. The column is loaded with 1.0 l $ZnCl_2$ 20 mmol/l solution and finally equilibrated with 3.0 l double distilled water and 3.0 l PBS containing 1 mol/l NaCl. The flow rate amounts to 1.5 cm/min.

The affinity chromatography eluate is diluted 1:2 with PBS and pumped through the column at a flow rate of 1.5 cm/min. IFN-beta is ca. 100% bound under these conditions. There follows a wash with 1.0 l PBS containing 1 ml/l NaCl and finally elution is carried out with a gradient of 0.5 l PBS containing 1 mol/l NaCl and 0.5 l PBS containing 1 mol/l NaCl together with 0.1 mol/l imidazole. In the wash ca. 15% to 20% of the interferon activity, in the elution ca. 70% of interferon activity are obtained. The recombinant IFN-beta presents at this stage a specific activity of 195 x 10^6 IU/mg protein.

The next stage is size exclusion chromatography (SEC). For this a chromatography column of 6 cm diameter is packed with 3.0 l Sephacryl® S-200 (Pharmacia). The gel bed height is ca. 90 cm. The column is equilibrated with 6.0 l PBS containing 0.1 mol/l NaCl. The flow rate is 0.5 cm/min. The application to the SEC column amounts to ca. 0.2 l of the eluate from the metal chelation column. The flow rate is 0.8 cm/min. The chromatography is performed with 3.0 l PBS containing 0.1 mol/l NaCl, with a

flow rate of 0.9 cm/min. In elution ca. 70% of the interferon activity is obtained. The recombinant IFN-beta has a specific activity of 220 x 10^6 IU/mg and a protein purity, determined by SDS-PAGE electrophoresis and silver staining, of > 99%.

The eluate of the size exclusion chromatography is assayed for identity (primary sequence, carbohydrate structures) and activity (biological assay, ELISA) and finally placed in formulation.

Example 2

Formulation of the recombinant IFN-beta

For formulation, the eluate from size exclusion chromatography, ca. 0.8 l with an interferon activity of 40 x 10^6 IU/ml and a protein content of 0.18 mg/ml is diluted with PBS to the desired activity per volume of, e.g., 30 x 10^6 IU/ml and mixed with 10 mg/ml human serum albumin as filler. This solution is sterile filtered through a 0.22 µm filter. From this formulation, 1 ml aliquots are added to DIN R2 vials and deep frozen at -50°C and finally lyophilised. The sealed, deep-frozen vials are stored at 15°C.

Example 3

Example 3 demonstrates the stability of preparations of various compositions containing recombinant IFN-beta from CHO cells with a purity of >99% in liquid formulation without stabilising additives.

Dissolved recombinant IFN-beta with 10^8 IU/ml and a protein content of 0.4 mg/ml is dialysed using the described buffer and finally diluted with the corresponding buffer to the required activity per volume.

The solution is then sterile filtered through a 0.22 µm filter. Finally 1 ml aliquots of this formulation are added to sterilised glass vials DIN 3 R (3 ml volume) and sealed

with a stopper Of each formulation a suitable number of vials is produced so that for each stability test a new sample is available.

Table 1

Formulation I: 0.2 M NaCl
0.05 M imidazole buffer pH = 7.5
70×10^6 IU/ml recombinant IFN-beta

Formulation II: 0.2 M NaCl
0.1 M sodium phosphate buffer pH = 7.5
30×10^6 IU/ml recombinant IFN-beta

Formulation III: 0.1 M NaCl
0.05 M sodium phosphate buffer pH = 7.5
30×10^6 IU/ml recombinant IFN-beta

No. of weeks' storage	Storage temp. °C	Formulation I	II	III
			Yield %	
1	-20	100	100	100
4	-20	100	100	100
50	-20	100	100	100
1	+15	97	100	98
4	+15	93	97	99
1	+25	92	95	98
4	+25	90	95	97

Table I shows that the high stability of the preparations according to the invention does not depend on the composition of the buffer.

Example 4

Example 4 demonstrates the stability of the preparations of various compositions containing recombinant IFN-beta from CHO cells with a purity of >99% in lyophilised form with human serum albumin as the filler. No stabilising additive was used.

Dissolved recombinant IFN-beta with an activity of 1.5×10^8 IU/ml and a protein content of 0.5 mg/ml are dialysed using the buffer mentioned without HSA and finally diluted to the desired activity per volume with the corresponding buffer with HSA.

The solution is then sterile filtered through a 0.22 µm filter and 1 ml aliquots added to sterilised glass vials DIN 3 R (3 ml volume) and lyophilised.

Of each formulation a suitable number of vials is produced so that for each stability test a new sample is available. After completion of the challenge test, the contents of one vial are added to 1 ml water for injections and the residual active substance determined by bioassay.

Table 2

Formulation A:	0.2	M NaCl
	0.05	M imidazole buffer pH = 7.5
	70×10^6	IU/ml recombinant IFN-beta
	18	mg/ml human serum albumin
Formulation B:	0.2	M NaCl
	0.1	M sodium phosphate buffer pH = 7.5
	3×10^5	IU/ml recombinant IFN-beta
	9	mg/ml human serum albumin
Formulation C:	0.2	M NaCl

0.1 M sodium phosphate buffer pH = 7.5

6×10^6 IU/ml recombinant IFN-beta

18 mg/ml human serum albumin

Formulation D:

0.1 M NaCl

0.05 M sodium phosphate buffer pH = 7.5

30×10^6 IU/ml recombinant IFN-beta

30 mg/ml human serum albumin

No. of weeks' storage	Storage temp. °C	Formulation A	B	C	D
		Yield %			
1	-20	100	100	100	100
4	-20	100	100	100	100
36	-20	100	100	100	100
1	+15	97	100	95	100
30	+15	93	95	96	97
1	+25	92	95	96	100
30	+25	90	93	96	93
1	+37	100	100	98	100
10	+37	90	90	92	90
25	+37	85	80	89	85
1	+51	100	100	98	95
5	+51	95	96	90	93
10	+51	90	90	88	88

The results of Table 2 show that the formulations according to the invention with recombinant IFN-beta enable a stable final formulation in physiological buffer at neutral pH without further additives for stabilisation. The serum albumin serves as a filler for the lyophilised protein, as the protein content of pure IFN-beta is only 10 to 200 µg.

The results also show that the formulations are also very stable in lyophilised form. The advantage of this stable formulation of the active substance lies in its long shelf-life. The storage temperature of +4 to +25°C is to be seen as a further advantage in that a chain of cold storage units , as is normal with interferon preparations, is superfluous.

Example 5

This example shows the stability of preparations of different compositions containing recombinant IFN-beta from CHO cells with a purity of >99% in lyophilised form and with human serum albumin as filler after reconstitution in 1 ml water for injections.

Dissolved recombinant IFN-beta with an activity of 1.5×10^8 IU/ml and a protein content of 0.6 mg/ml is dialysed using the buffer mentioned without HSA and finally diluted with the corresponding buffer with HSA to the required activity per volume.

The solution is then sterile filtered through a 0.22 µm filter and 1 ml aliquots added to sterilised glass vials DIN 3 R (3 ml volume), stoppered and lyophilised.

Of each formulation a suitable number of vials is produced so that for each stability test a new sample is available. After a storage period of 4 weeks at +4°C the lyophilisate is dissolved in 1 ml water for injects. This solution is then used for stability testing.

Table 3

Formulation A:	0.2	M NaCl
	0.05	M imidazole buffer pH = 7.5

7×10^6 IU/ml recombinant IFN-beta
18 mg/ml human serum albumin

Formulation B: 0.2 M NaCl
0.1 M sodium phosphate buffer pH = 7.5
3×10^6 IU/ml recombinant IFN-beta
9 mg/ml human serum albumin

Formulation C: 0.2 M NaCl
0.1 M sodium phosphate buffer pH = 7.5
6×10^6 IU/ml recombinant IFN-beta
18 mg/ml human serum albumin

Formulation D: 0.1 M NaCl
0.05 M sodium phosphate buffer pH = 7.5
30×10^6 IU/ml recombinant IFN-beta
30 mg/ml human serum albumin

No. of weeks' storage	Storage temp. °C	Formulation A	B	C	D
		Yield %			
1	+15	97	100	95	95
30	+15	93	95	96	97
1	+25	92	95	96	95
14	+25	90	93	96	93
1	+37	100	100	98	98
15	+37	90	90	92	90
30	+37	85	80	89	85
1	+51	90	95	95	85

The high stability of the preparations according to the invention are, as Table 3 shows, not influenced by the composition of the buffer. This shows that the preparations according to the invention are very stable after lyophilisation and reconstitution. The advantage of this stable formulation of the active substance is demonstrated advantageously above all in the case of infusions over a long period at room temperature.

Example 6

This example shows the decreased stability of preparations containing recombinant IFN-beta from CHO cells with a purity of >99% after treatment with sialidase. This enzyme causes defined sialic acid present to be degraded enzymatically.

1 mg recombinant IFN-beta with a purity of >99% in a 0.05 mol/l sodium acetate buffer solution, pH = 5.5, is mixed with 0.02 mg neuraminidase (sialidase) ex Boehringer Mannheim and incubated at 25°C for 6 hours. The sialic acid liberated is determined by anion exchange chromatography. With a remaining sialic acid content of ca 60 to 70% ultrafiltration with an exclusion limit of 30 000 Daltons is performed. The neuraminidase is retained in the concentrate, while the recombinant IFN-beta is present in the permeate. After dialysis using formulation buffers II and III, the stability is determined as described in Example 1.

Table 4

Formulation II:	0.2	M NaCl
	0.1	M sodium phosphate buffer pH = 7.5
	30×10^6	IU/ml recombinant IFN-beta
Formulation III:	0.2	M NaCl
	0.1	M sodium phosphate buffer pH = 7.5

30×10^6 IU/ml recombinant IFN-beta			
No. of weeks' storage	Storage temp. °C	Formulation II	III
		Yield %	
1	+15	80	85
30	+15	35	42
1	+25	55	60
14	+25	10	15

Example 6 demonstrates the relationship between the degree of sialisation and the stability of recombinant IFN-beta.

The markedly lower sialic acid content of ca. 60 to 70% leads to greater instability of IFN-beta. With the reduction of the sialic acid content there is a parallel reduction in the solubility and the IFN-beta tends to cloud and precipitate out.

Example 7

In this example the composition of the carbohydrate content of IFN-beta, produced in FS4 cells in cell culture and the recombinant IFN-beta according to the invention produced in CHO cell cultures was assayed. Unless otherwise stated, the method of determining the carbohydrate content is that described in The Journal of Biological Chemistry, Vol 262, no.30, October 25, 1987, page 25. The primary structures of the recombinant IFN beta from CHO cells and the IFN-beta from FS4 cells are identical. By comparison with natural IFN-beta from fibroblasts (FS4), the recombinant IFN-beta from CHO cells according to the invention shows a markedly higher proportion in the range of the triantennal and more highly sialised structures. The sialisation level in natural IFN-beta is ca. 70%, in the recombination IFN-beta according to the invention it is over 90%.

The recombinant IFN-beta produced as described here is surprisingly markedly more effective clinically than natural IFN-beta, as the following examples show.

Example 7

Clinical testing

Patient:	male, 47 years of age, homosexual
Disorder	AIDS, classification CDC IV/WR 5 Kaposi's sarcoma of the entire integument
Treatment:	3×10^6 IU IFN-beta according to the invention referred to above, subcutaneously 3 x per week for 5 weeks plus zidovudine 1000 mg/d
Result:	Reduction of the Kaposi's sarcoma in size and number
Treatment	3×10^6 IU IFN-beta according to the invention referred to above, subcutaneously 3 x per week for 6 weeks plus zidovudine 500 mg/d
Result:	Enlargement of the Kaposi's sarcoma
Treatment:	3×10^6 IU IFN-beta according to the invention referred to above, subcutaneously 3 x per week continuously plus zidovudine 500 mg/d for approx. 6 weeks
Result:	Diminution of the Kaposi's sarcoma in size and number.

Example 8

Clinical testing

Patient:	male, 43 years of age, homosexual
Disorder	AIDS, classification CDC IV/WR 5 Kaposi's sarcoma of the entire integument with involvement of the oral mucosa
Treatment:	3×10^6 IU IFN-beta according to the invention referred to above, subcutaneously 3 x per week for 9 weeks plus zidovudine 500 mg/d
Result:	Diminution of the Kaposi's sarcoma in size and number
Treatment	3×10^6 IU highly purified natural IFN-beta subcutaneously 3 x per week for 6 weeks plus zidovudine 500 mg/d
Result:	Onset of new Kaposi's sarcoma
Treatment:	3×10^6 IU IFN-beta according to the invention referred to above, subcutaneously 3 x per week continuously plus zidovudine 500 mg/d for ca. 6 weeks
Result:	Diminution of the Kaposi's sarcoma in size and number.

Claims

1. Recombinant human-IFN-beta from CHO-cell cultures, characterised in that it contains a proportion of diantennal oligosaccharide structures amounting to at least 70%, triantennal oligosaccharide structures amounting to at least 20% and tetraantennal oligosaccharide structures amounting to between 0 and 5% and a sialic acid content of at least 90%.

2. Recombinant human IFN-beta according to Claim 1, characterised in that the proportion of diantennal oligosaccharide structures amounts to at least 75%.

3. Recombinant human IFN-beta according to Claim 1 or 2, characterised in that the proportion of triantennal oligosaccharide amounts to at least 25%.

4. Recombinant human-IFN-beta according to Claims 1 to 3, characterised in that the triantennal structures may comprise at least one N-acetyllactosamine repeat.

5. Recombinant human-IFN-beta according to Claims 1 to 4, characterised in that the triantennal structure is linked at positions 1->4 and/or 1->6.

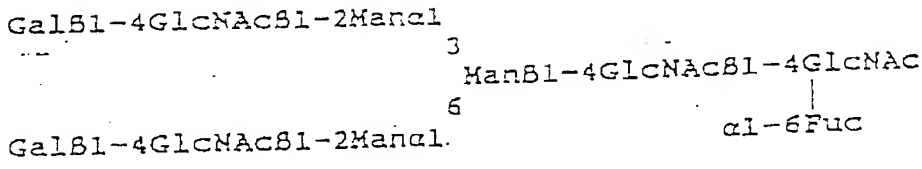
6. Recombinant human-IFN-beta according to Claims 1 to 5, characterised in that the proportion of tetraantennal oligosaccharide structures amounts to 0.5 to 3%.

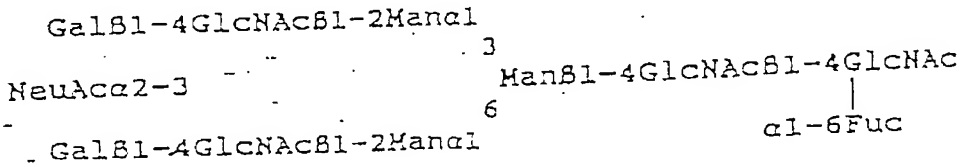
7. Recombinant human-IFN-beta according to one of Claims 1 to 6, characterised in that the sialic acid content is composed of N-acetylneuraminic acid and N-glycolylneuraminic acid.

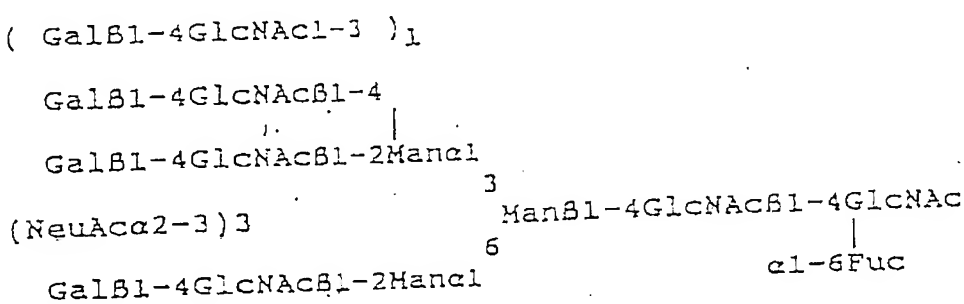
8. Recombinant human-IFN-beta according to Claim 7, characterised in that the proportion of N-acetylneuraminic acid present is between 90 and 100% and the proportion of N-glycolylneuraminic acid amounts to 0 to 10%.

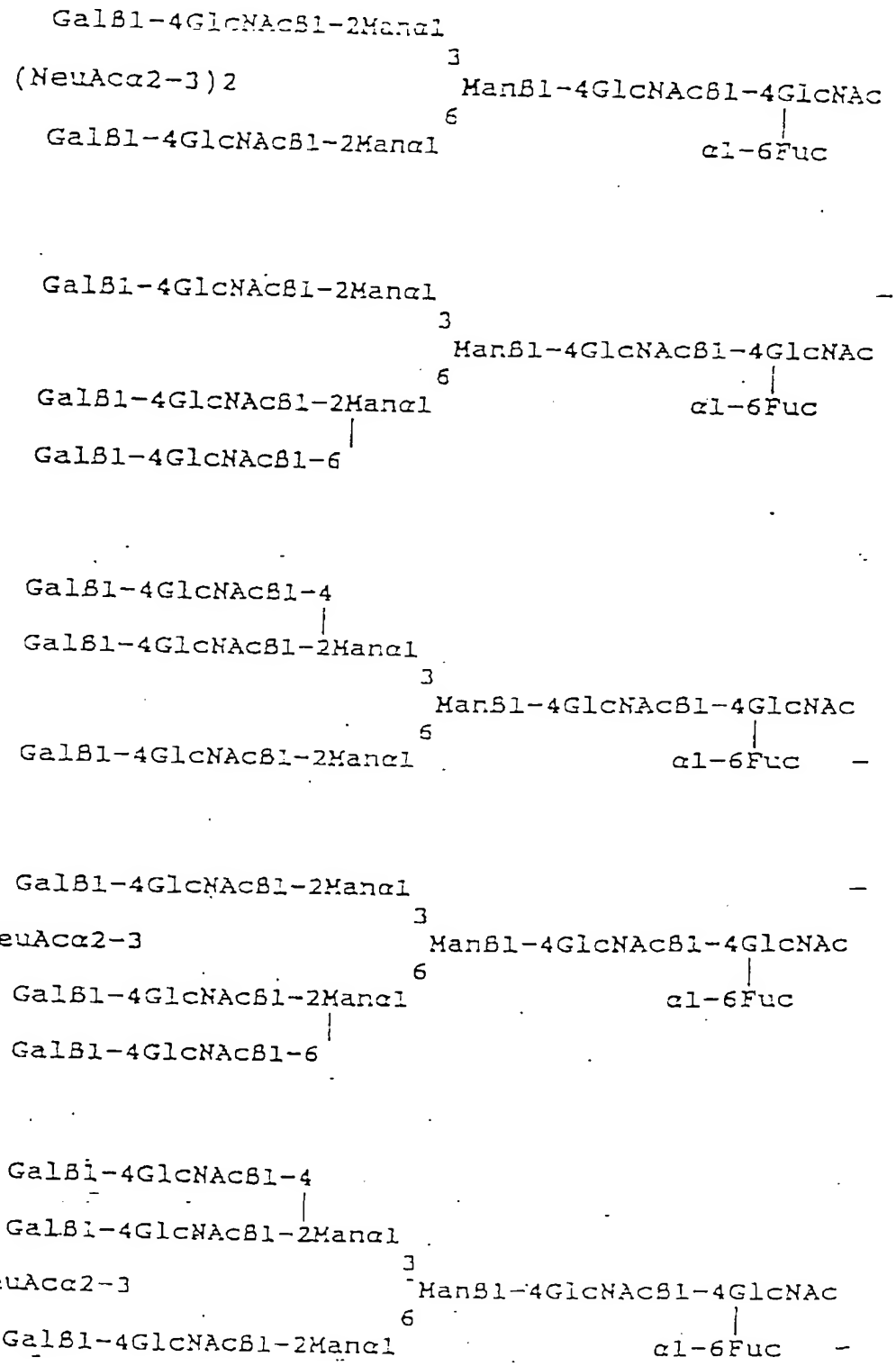
9. Recombinant human-IFN-beta according to Claims 1 to 8, characterised in that the recombinant IFN-beta also contains fucose at a rate of at least 85%, preferably 90% and most preferably > 95%.

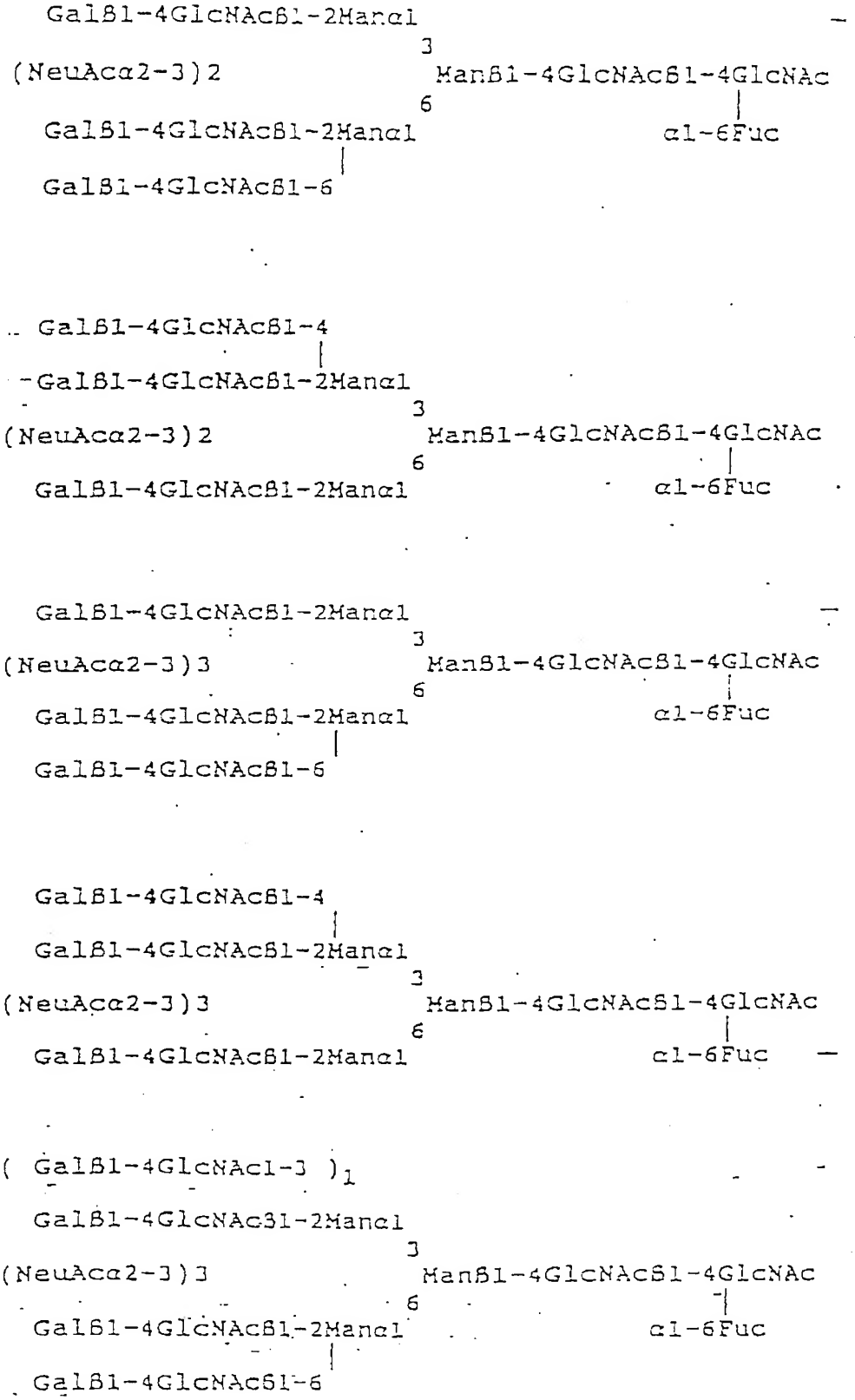
10. Recombinant human-IFN-beta according to Claims 1 to 9, characterised in that at least one oligosaccharide structure corresponds to one of the following formulae:








Galβ1-4GlcNAcβ1-2Manα1
3
(NeuAcα2-3)2
Manβ1-4GlcNAcβ1-4GlcNAc
6
Galβ1-4GlcNAcβ1-2Manα1
α1-6Fuc
Galβ1-4GlcNAcβ1-2Manα1
3
Manβ1-4GlcNAcβ1-4GlcNAc
6
Galβ1-4GlcNAcβ1-2Manα1
α1-6Fuc
Galβ1-4GlcNAcβ1-6
Galβ1-4GlcNAcβ1-4
Galβ1-4GlcNAcβ1-2Manα1
3
Manβ1-4GlcNAcβ1-4GlcNAc
6
Galβ1-4GlcNAcβ1-2Manα1
α1-6Fuc
Galβ1-4GlcNAcβ1-2Manα1
3
NeuAcα2-3
Manβ1-4GlcNAcβ1-4GlcNAc
6
Galβ1-4GlcNAcβ1-2Manα1
α1-6Fuc
Galβ1-4GlcNAcβ1-6
Galβ1-4GlcNAcβ1-4
Galβ1-4GlcNAcβ1-2Manα1
3
NeuAcα2-3
Manβ1-4GlcNAcβ1-4GlcNAc
6
Galβ1-4GlcNAcβ1-2Manα1
α1-6Fuc



in which NeuAc represents N-glycolylneuraminic acid

11. Recombinant human-IFN-beta according to Claims 1 to 10, characterised in that it has a specific activity of at least 200 x 10^6 IU/mg.

12. Method for the production of recombinant human-IFN-beta according to Claims 1 to 11, characterised in that an aqueous solution of human-IFN-beta in an impure state is obtained by liquid/liquid phase extraction, then colour affinity chromatography performed, and finally metal chelation chromatography, followed by size-exclusion chromatography, in which the chromatographic stages are carried out in this order.

13. Method according to Claim 12, characterised in that the liquid/liquid phase extraction is carried out with an aqueous two-phase system based on polyalkyleneglycol/dextran or polyalkyleneglycol/salt.

14. Method according to Claim 13, characterised in that the polyalkyleneglycol used is polyethyleneglycol with a molecular weight of 1 000 to 6 000 or polypropyleneglycol with a molecular weight of 1 500 to 4 000.

15. Method according to Claim 13, characterised in that the salt used is NaCl, LiCl, NaI, KI, NA_2SO_4, NA_2HPO_4, K_2HPO_4,.K_2SO_4, NaH_2PO_4, KCl, NH_4Cl, $(NH_4)_2SO_4$, sodium citrate or sodium oxalate.

16. Method according to Claim 13, characterised in that the dyestuff used in affinity chromatography is Cibacron blue F 3 GA.

17. Method according to Claim 11, characterised in that the ions used in metal chelation chromatography are Cu^{2+}, Zn^{2+}, Co^{2+} or Ni^{2+}.

18. Method according to Claim 12, characterised in that, for the size exclusion

chromatography, separation media are used which have a separation range of 1 000 to 600 000 daltons, preferably Sephadex® G150, Sephadex® G150 superfine, Sephacryl® S-200 High Resolution, Superose 12 prep grade or TSK-SW 3000.

19. Method according to Claims 13 and 16, characterised in that the elution in affinity chromatography is carried out using PBS containing 10 to 70% by weight of ethylene glycol and/or 20 to 50% by weight of propylene glycol.

20. Method according to Claims 13 and 17, characterised in that the elution in metal chelation chromatography is carried out using a competitive substance such as imidazole, histidine, glycine or NH_4Cl or a pH gradient of around pH 7 to around pH2 or by means of isocratic elution with descending pH.

21. Method according to Claims 13 and 18, characterised in that the elution in size exclusion chromatography is carried out with PBS containing 0 to 0.5 mol/l NaCl.

22. Pharmaceutical preparation containing recombinant human-IFN-beta according to Claims 1 to 13, in a compound formulated with the usual vehicles and excipients.

23. Pharmaceutical preparation according to Claim 22, characterised in that it contains at least 25 x 10^6 IU/ml IFN-beta.

24. Pharmaceutical formulation according to Claim 22 or 23, characterised in that it is available in forms appropriate for topical or parenteral administration.

25. Pharmaceutical formulation according to Claim 23 characterised in that it is available in the form of a gel, emulsion or ointment.

26. Pharmaceutical preparation according to Claim 23, characterised in that it is available in the form of a lyophilisate, solution or infusion.